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Role of Adiponectin and Inflammation in Insulin Resistance of Mc3r and Mc4r Knockout Mice

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Abstract

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Objective: To investigate the involvement of hypoadiponectinemia and inflammation in coupling obesity to insulin resistance in melanocortin-3 receptor and melanocortin-4 receptor knockout (KO) mice (Mc3/4rKO).

Research Methods and Procedures: Sera and tissue were collected from 6-month-old Mc3rKO, Mc4rKO, and wild-type C57BL6J litter mates maintained on low-fat diet or exposed to high-fat diet (HFD) for 1 or 3 months. Inflammation was assessed by both real-time polymerase chain reaction analysis of macrophage-specific gene expression and immunohistochemistry.

Results: Mc4rKO exhibited hypoadiponectinemia, exacerbated by HFD and obesity, previously reported in murine models of obesity. *Mc4r* deficiency was also associated with high levels of macrophage infiltration of adipose tissue, again exacerbated by HFD. In contrast, Mc3rKO exhibited normal serum adiponectin levels, irrespective of diet or obesity, and a delayed inflammatory response to HFD relative to Mc4rKO.

Discussion: Our findings suggest that severe insulin resistance of Mc4rKO fed a HFD, as reported in other models of obesity such as leptin-deficient (Lep^{ob}/Lep^{ob}) and $KK\text{-}A^{y}$ mice, is linked to reduced serum adiponectin and high levels of inflammation in adipose tissue. Conversely, maintenance of normal serum adiponectin may be a factor in the relatively mild insulin-resistant phenotype of severely obese Mc3rKO. Mc3rKO are, thus, a unique mouse model where obesity is not associated with reduced serum adiponectin levels. A delay in macrophage infiltration of adipose tissue of Mc3rKO during exposure to HFD may also be a factor contributing to the mild insulin resistance in this model.

Key words: hypothalamic neurotransmitters, macrophages, dietary fat, inflammation, insulin resistance

Introduction

An increasing occurrence of obesity has been associated with a parallel rise in the incidence of metabolic syndrome, a cluster of diseases with insulin resistance as the defining feature (1). The pathogenesis of obesity-related insulin resistance is poorly understood. Several mechanisms have been proposed to explain the increased risk of insulin resistance, including an imbalance of fat intake and oxidation associated with defects in oxidative metabolism (2), hypothalamic dysfunction (3), and oxidative stress and inflammation in adipocytes (4). It is likely that no single mechanism will explain obesity-related insulin resistance and that insulin resistance and type 2 diabetes represent the culmination of a cascade of events.

The axes involving factors secreted from the gut and adipose tissue that act on melanocortin neurons in the central nervous system have been the most intensively investigated homeostatic system involved in energy homeostasis and insulin resistance (3,5). Leptin deficiency is associated with insulin resistance and steatosis (6–9). Intracerebroventricular administration of leptin improves hepatic insulin sensitivity, independently of effects on food intake (10).

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Administration of non-selective melanocortin agonists intracerebroventricularly also improves insulin sensitivity independently of reduced food intake (11). The selective roles of two melanocortin receptors expressed in the central nervous system (Mc3r, Mc4r) in energy homeostasis and insulin resistance have been investigated using knockout (KO)[1] mice (Mc3rKO, Mc4rKO). The initial analysis of obesity and diabetes (diabesity) in melanocortin receptor KO mice suggested a role for the Mc4r in the regulation of insulin action (12,13). Analysis of the phenotype of melanocortin receptor KO mice backcrossed onto the C57BL/6J (B6) background also suggests that insulin resistance is most severe for Mc4rKO (14). The mechanisms explaining the differential effects of melanocortin receptor genotype on insulin resistance in the obese state are unclear.

Altered metabolic state of adipose tissue has also been linked to the development of obesity and insulin resistance. Serum levels of the adipocyte-derived hormone adiponectin are reduced in states of obesity and diabetes (15). Administration of adiponectin protein inhibits endogenous glucose production in the liver and improves obesity-associated insulin resistance (16,17). Macrophage infiltration of adipose tissue has also been observed in obesity-related insulin resistance (18,19), possibly mediated by increased levels of the chemokine monocyte chemoattractant protein (MCP)-1 (20).

The objective of these studies was to investigate adipocytokine production and adipose tissue inflammation in Mc3rKO and Mc4rKO. We also measured adipocyte size because human obesity and insulin resistance are associated with an increase in both the number and size of adipocytes (21). Our data suggest that differences in the effects of obesity on serum adiponectin and the severity of inflammation may contribute to the observed differences in hyperinsulinemia in severely obese Mc3rKO and Mc4rKO.

Research Methods and Procedures

Experimental Animals

The Pennington Biomedical Research Center Institutional Animal Care and Use Committee approved all experiments. Mc3rKO, Mc4rKO, and wild-type mice (WT) B6 littermates were obtained from mating heterozygote parents, as described previously (22,23). Mice were housed on a 12-hour light/dark period (lights on, 6 AM to 6 PM) and fed standard chow from weaning to ~12 to 14 weeks of age. Mice were then fed either purified low-fat diet (LFD; Research Diets 12450B, Research Diets, New Brunswick, NJ; 10% kJ/fat; 10 to 12 of each genotype) or high-fat diet (HFD; Research Diets 12492, 60% kJ/fat; five to six of each genotype) for 3 months. Mice were sacrificed at 24 to 26 weeks of age. Four weeks before sacrifice, half of the LFD group were fed HFD ad libitum. Adiposity (percentage body fat) was estimated by measuring fat mass and fat-free mass by nuclear magnetic resonance (Bruker Mice Minispec NMR Analyzer; Bruker Optics Inc., Billerica, MA), validated against DXA and standard chemical composition analysis (24).

Gene Expression

Total RNA from retroperitoneal adipose tissue was isolated using TRI Reagent (Molecular Research Centre Inc., Cincinnati, OH), quantitated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and reverse transcribed using the Superscript III reverse transcription system (Invitrogen, Carlsbad, CA). Oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Quantitation of target gene mRNA using cyclophilin B as a reference was performed in 384-well plates using SYBR Green or Taqman Universal PCR Master Mix (Applied Biosystems) and an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Sequences of primers and probes used for expression analyses were: cyclophilin B, sense, 5′-ggtggagagcaccaagacaga-3′, antisense, 5′-gccggagtcgacaatgatg-3′; probe, 5′-agccgggacaagccactgaaggat-3′; adiponectin (*Acrp30*; adipocyte complement-related protein of 30 kDa), sense, 5′-gctcctgctttggtccctccac-3′, antisense, 5′-gcccttcagctcctgtcattcc-3′; epidermal growth factor-like module-containing, mucin-like, hormone receptor-like sequence (*Emr1*; epidermal growth factor-like module containing, mucin-like, hormone receptor-like 1), sense 5′-ctttggctatgggcttccagtc-3′, antisense, 5′-gcaaggaggacagagtttatcgtg-3′; CD68 antigen (*Cd68*) sense, 5′-cttcccacaggcagcacag-3′, antisense, 5′-aatgatgagaggcagcaagag-3′; and *Mcp1,* sense, 5′-ttggctcagccagatgca-3′, antisense, 5′-ccagcctactcattgggatca-3′. The 5-carboxyfluorescein-labeled probe was synthesized by Applied Biosystems.

Serum Assays

Total serum insulin and leptin (Crystal Chem, Downer's Grove, IL) and adiponectin (Linco Research, St. Charles, MO) were measured by enzyme-linked immunosorbent assay performed in duplicate.

Gel Electrophoresis and Immunoblotting

Serum adiponectin immunoreactivity was examined under non-denaturing conditions to investigate the effect of obesity on the low-molecular weight, middle-molecular weight (MMW), and high-molecular weight (HMW) multimers (25). Proteins were separated in 7.5% polyacryl-

[1] Nonstandard abbreviations: KO, knockout; Mc3rKO, Mc3r KO mice; Mc4rKO, Mc4r KO mice; MCP, monocyte chemoattractant protein; WT, wild type mice; LFD, low-fat diet; HFD, high-fat diet; *Acrp30,* adipocyte complement-related protein of 30 kDa; *Emr1,* epidermal growth factor-like module containing, mucin-like, hormone receptor-like 1; MMW, middle molecular weight; HMW, high molecular weight; WAT, white adipose tissue; MW, molecular weight.

amide gels that either contained sodium dodecyl sulfate or were devoid of sodium dodecyl sulfate. The gels were transferred to nitrocellulose (Bio-Rad, Hercules, CA) in 25 mM Tris, 192 mM glycine, and 20% methanol. After transfer, the membrane was blocked in 4% milk for 1 hour at room temperature. Results were visualized with horseradish peroxidase-conjugated secondary antibodies (Sigma, St. Louis, MO) and enhanced chemiluminescence (Pierce, Rockford, IL). An adiponectin polyclonal antibody was purchased from Affinity Bioreagents (Golden, CO) and used at a dilution of 4 μg/mL.

Fat Cell Size Analysis

Fat cell size was determined as previously described (26). Briefly, adipose tissue was fixed in osmium tetrachloride/collidine-HCl after disassociation by urea digestion. Cells were counted on a Multisizer-3 (Beckman Coulter, Fullerton, CA) using a 400-μm aperture (dynamic linear range, 12 to 320 μm).

Immunohistochemistry

Formalin-fixed fat depot samples were processed, embedded in paraffin, and sectioned at 5 μm. Dewaxed sections were treated with 3% H_2O_2 in methanol (Sigma) for 30 minutes to block endogenous peroxidases followed by normal horse serum to reduce non-specific staining. Consecutive sections were incubated overnight (4 °C) with the following monoclonal primary antibodies: anti-mouse Mac-2 (1:3000; Cedarlane Laboratories, Ontario, Canada) and anti-mouse F4/80 (1:50; Serotec, Oxford, U.K.). Antibody binding was detected with the avidin:biotinylated enzyme complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA). Peroxidase activity was revealed using 3.3′-diaminobenzidine (Sigma) as a substrate. Two types of controls were performed: the primary antibody was omitted during the immunostaining procedure, and the primary antibody was substituted with non-specific Ig G during the procedure. Sections were counterstained with hematoxylin.

Morphometry

Sections were visualized with a Zeiss microscope (Axioskop 40; Carl Zeiss GmbH, Jena, Germany) using a Plan-Neofluor ×10 objective and photographed with a Kodak digital camera (DC290 Zoom; Eastman Kodak, Rochester, NY). Quantitative analyses of immunopositive cells were made using MetaMorph software (Molecular Devices Corp., Sunnyvale, CA). For macrophage quantitation, adipocytes and macrophages were counted from five fields, and the results are presented as the mean of the five fields.

Statistics

Data presented are mean ± standard error. Statistical analyses were performed using JMP IN 5.1 statistical software (SAS Institute, Inc., Cary, NC). Differences between genotypes were assessed by ANOVA within dietary treatment groups for normally distributed data and Tukey-Kramer honestly significant difference post hoc test. Significance was assumed for p values <0.05.

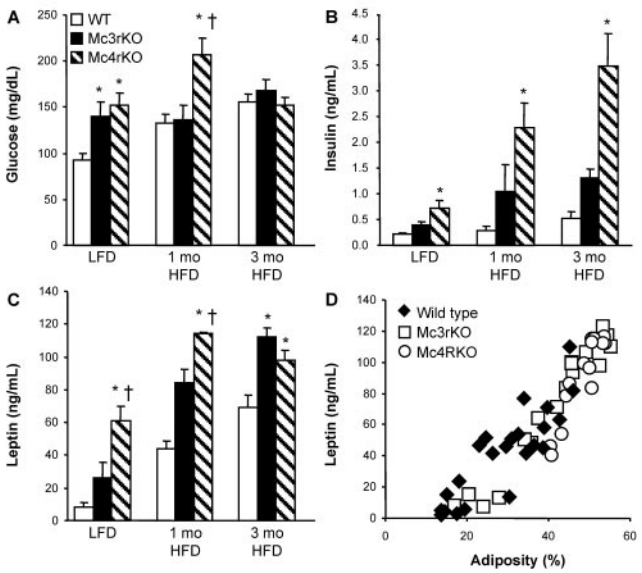

Figure 1: Fasting glucose (A), insulin (B), and serum leptin (C) in WT, Mc3rKO, and Mc4rKO maintained on LFD or HFD for either 1 or 3 months. (D) Relationship between serum leptin and adiposity (percentage body fat) in WT, Mc3rKO, and Mc4rKO. * $p <$ 0.05 compared with WT. † $p < 0.05$ compared with Mc3rKO.

Results

Body Composition and Serum Analysis

Fasting insulin, adiponectin, and inflammation of adipose tissue were examined in 6-month-old mice fed LFD or exposed to HFD for 1 or 3 months. The LFD and 3-month HFD body weight and adiposity data have been published previously (14). Adiposity data of female WT, Mc3rKO, and Mc4rKO from that report are incorporated as part of the data set for Figure 1D. After 3 months on HFD, there was no difference in adiposity between Mc3rKO and Mc4rKO [WT, 38.2 ± 1.6%; Mc3rKO, 54.0 ± 2.2%; Mc4rKO, 50.1 ± 2.2%; $p < 0.05$; WT < Mc3rKO, Mc4rKO (14)]. Mc3rKO and Mc4rKO were moderately hyperglycemic compared with WT on LFD (Figure 1A). Mc4rKO fed HFD for 1 month exhibited the most severe hyperglycemia (fasting glucose >200 mg/dL), which was not observed after 3 months of exposure to HFD (Figure 1A), possibly due to a compensatory increase in the production of insulin by β-cells (Figure 1B). Mc4rKO displayed hyperinsulinemia compared with WT irrespective of diet, whereas Mc3rKO did not exhibit significantly higher fasting insulin levels compared with WT (Figure 1B). Serum leptin levels were increased in Mc4rKO on LFD and after 1 month of expo-

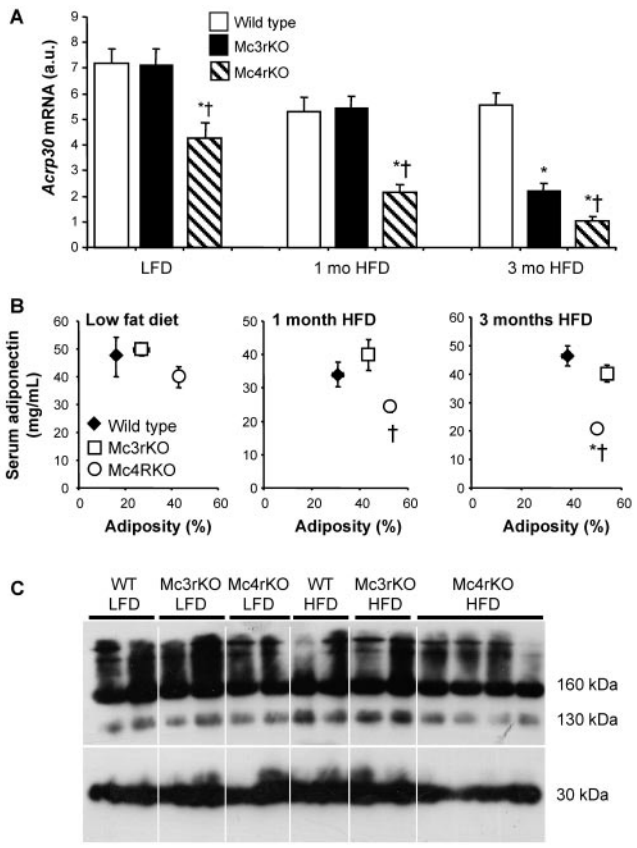

Figure 2: Serum adiponectin and adiponectin gene expression are reduced in Mc4rKO. (A) Adiponectin gene expression in retroperitoneal WAT from WT, Mc3rKO, and Mc4rKO maintained on LFD or HFD for either 1 or 3 months. (B) Relationship between serum adiponectin and adiposity separated by dietary treatment. (C) The differences in patterns of adiponectin oligomeric complex distribution in WT, Mc3rKO, and Mc4rKO on LFD or HFD for 3 months. Mouse serum samples (1 μL) from mice were separated by gel electrophoresis, transferred to nitrocellulose, and subjected to Western blot analysis. * $p < 0.05$ compared with WT. † $p < 0.05$ compared with Mc3rKO.

sure to HFD compared with WT and Mc3rKO, with 3 months of HFD elevating serum leptin in Mc3rKO to similar levels as Mc4rKO (Figure 1C). Overall, serum leptin correlated significantly with adiposity when all mice were grouped ($r^2 = 0.86$, $p < 0.001$, Figure 1D).

Hypoadiponectemia Associated with Obesity in Mc4rKO But Not in Mc3rKO

Expression of adiponectin mRNA (*Acrp30*) in retroperitoneal white adipose tissue (WAT) of WT, Mc3rKO, and Mc4rKO was affected by exposure to HFD, decreasing with long-term exposure to HFD irrespective of genotype (Figure 2A). *Acrp30* mRNA was significantly lower in Mc4rKO compared with WT and Mc3rKO, irrespective of diet (Figure 2A). Long-term exposure to HFD reduced *Acrp30* mRNA in Mc3rKO compared with WT mice. Two-way ANOVA revealed significant effect of diet and genotype on *Acrp30* gene expression ($p < 0.001$). Serum adiponectin levels generally correlated with *Acrp30* mRNA. However, although *Acrp30* mRNA was significantly reduced in Mc3rKO compared with WT after 3 months on HFD, there was no significant difference in serum adiponectin (Figure 2B). Moreover, although *Acrp30* mRNA was significantly lower in Mc4rKO related to WT and Mc3rKO on LFD (Figure 2A), this was not associated with a statistically significant reduction in serum adiponectin (Figure 2B).

Several different molecular weight (MW) species of adiponectin are observed in serum, correlating with the formation of trimers, hexamers, and multimeric complexes, with HMW forms having more important effects on insulin sensitivity status (27). The MW forms of adiponectin were analyzed using non-denaturing polyacrylamide gel electrophoresis and Western blot. Mc4rKO demonstrated reduced levels of the higher molecular weight isoforms of adiponectin, whereas Mc3rKO exhibited normal levels of the MMW and HMW forms of adiponectin (Figure 2C).

Increase in Expression of Macrophage-Specific Genes in Mc4rKO and by HFD

Macrophages in adipose tissue were analyzed using the expression of two macrophage-specific genes: *Cd68*, a macrophage-specific transmembrane protein; and *Emr1*, the F4/80 antigen. On LFD, Mc4rKO exhibited a modest but statistically significant increase in the expression of *Cd68* and *Emr1* compared with WT and to Mc3rKO for *Emr1* (Figure 3, A and B). Short-term exposure to HFD greatly increased expression of both genes to a far greater extent in Mc4rKO than in Mc3rKO; however, after 3 months of exposure to HFD, we observed significantly elevated levels of *Cd68* and *Emr1* mRNA in Mc3rKO (Figure 3, A and B). Similar results were also observed for expression of *Mcp1*, which was elevated in Mc4rKO on LFD compared with WT and was greatly increased by HFD (Figure 3C). After 3 months of exposure to HFD, *Mcp1* expression in Mc4rKO was reduced compared with Mc3rKO but remained greater than WT (Figure 3C).

Emr1 and *Cd68* gene expression data were confirmed by visualizing macrophages present in retroperitoneal WAT of WT, Mc3rKO, and Mc4rKO using an antibody targeted to the macrophage-specific antigen Mac-2 (Figure 4A). Overall, the ratio of macrophages per number of adipocytes visualized using immunohistochemistry supported the *Cd68* and *Emr1* gene expression data and was highest in Mc4rKO, irrespective of diet (Figure 4, B to D). Mc4rKO exhibited pronounced inflammation of adipose tissue, which was exacerbated by HFD, whereas Mc3rKO demonstrated an attenuated and delayed inflammatory response to HFD.

The interaction among fat mass, inflammation, and hyperinsulinemia is further illustrated in Figure 5. Expression

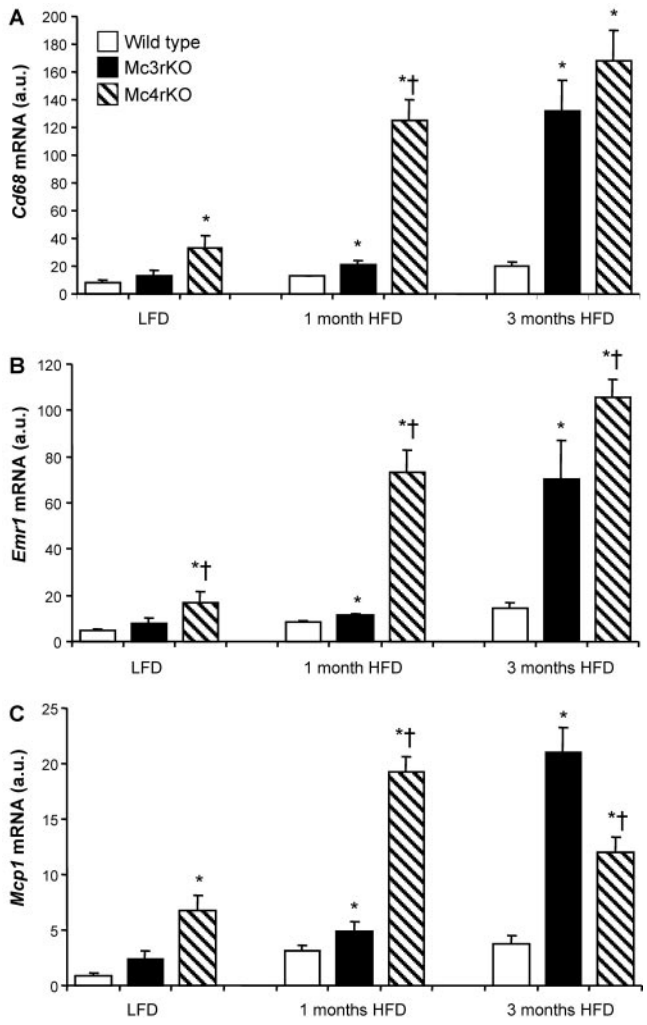

Figure 3: Expression of macrophage-specific genes is associated with adiposity. Retroperitoneal white adipose tissue expression of the macrophage markers *Cd68* (A), *Emr1* (B), and the chemokine *Mcp1* (C) in WT, Mc3rKO, and Mc4rKO exposed to LFD or HFD for either 1 or 3 months. * $p < 0.05$ compared with WT. † $p < 0.05$ compared with Mc3rKO.

of *Cd68* mRNA remains low in adipose tissue of all mice below a threshold level of adiposity (~45%), and when adiposity exceeds this threshold, *Cd68* expression is greatly increased (Figure 5A). Similar relationships were also observed between adiposity and *Emr1* and *Mcp1* expression (data not shown). The association between hyperinsulinemia and *Cd68* expression with adiposity is similar (Figure 5B). When a particular level of adiposity is achieved, evidence of macrophage infiltration and hyperinsulinemia becomes apparent.

Fat Cell Size Correlation with Obesity in Obese Melanocortin Receptor-Deficient Mice

Exposure to HFD for 1 or 3 months was associated with adipocyte hypertrophy in WT without significantly altering total adipocyte number (Table 1). On LFD, Mc3rKO and Mc4rKO exhibited adipocyte hypertrophy, although total fat cell number was not significantly different (Table 1). Exposure to HFD for 1 month induced marked adipocyte hypertrophy and hyperplasia in Mc4rKO, while increasing adipocytes size in Mc3rKO. Three months on HFD resulted in both Mc3rKO and Mc4rKO exhibiting adipocyte hyperplasia, compared with WT (Table 1). Fat cell size correlated strongly with adiposity when all mice were combined ($r^2 = 0.83$, $p < 0.001$; data not shown), but was not strongly associated with *Cd68* mRNA (not shown). Although smaller adipocytes size was associated with a low inflammatory state, adipocyte hypertrophy was not a prerequisite for macrophage infiltration in Mc4rKO (data not shown).

Discussion

The hypothalamic melanocortin system regulates insulin secretion and sensitivity independently of effects on body weight (11,13). Central administration of non-selective melanocortin receptor antagonists is associated with insulin resistance (11). The analysis of diabesity in Mc3rKO and Mc4rKO involving mice on outbred backgrounds (129;B6) suggested that loss of Mc4r has a more significant impact on both obesity and insulin resistance (12,22,28). These results were supported by our analysis of insulin sensitivity in age- and weight-matched Mc3rKO and Mc4rKO backcrossed onto C57BL/6J (14), a strain frequently used to investigate obesity and insulin resistance (for review, see 29). The objective of the current study was to further investigate mechanisms explaining the differential effects of diet and obesity on insulin resistance in Mc3rKO and Mc4rKO. Overall, the current data are consistent with previously published data demonstrating the relative importance of Mc3r and Mc4r in maintaining body weight and insulin sensitivity (14). The novel data reported herein suggest that differences in the effect of obesity on serum adiponectin, and to a lesser degree the inflammatory state, may at least partially explain the mild insulin-resistant phenotype of Mc3rKO compared with Mc4rKO.

Hotamisligil et al. (30) suggested that tumor necrosis factor α produced by adipocytes and macrophages contributes to the development of insulin resistance in the obese state, prompting the hypothesis that insulin resistance may be prevented or attenuated by inhibition of inflammation. The relationship between macrophage infiltration of adipose tissue and the development of insulin resistance has been further investigated in recent years (for review, see 4). The list of secreted factors produced by adipocytes contributing to inflammation and insulin resistance has expanded to include interleukin-6, resistin, MCP-1, leptin, and adiponectin, among several others. The role of inflammatory factors in the progression of obesity and metabolic syndrome in Mc4rKO and Mc3rKO had not been explored. In the current

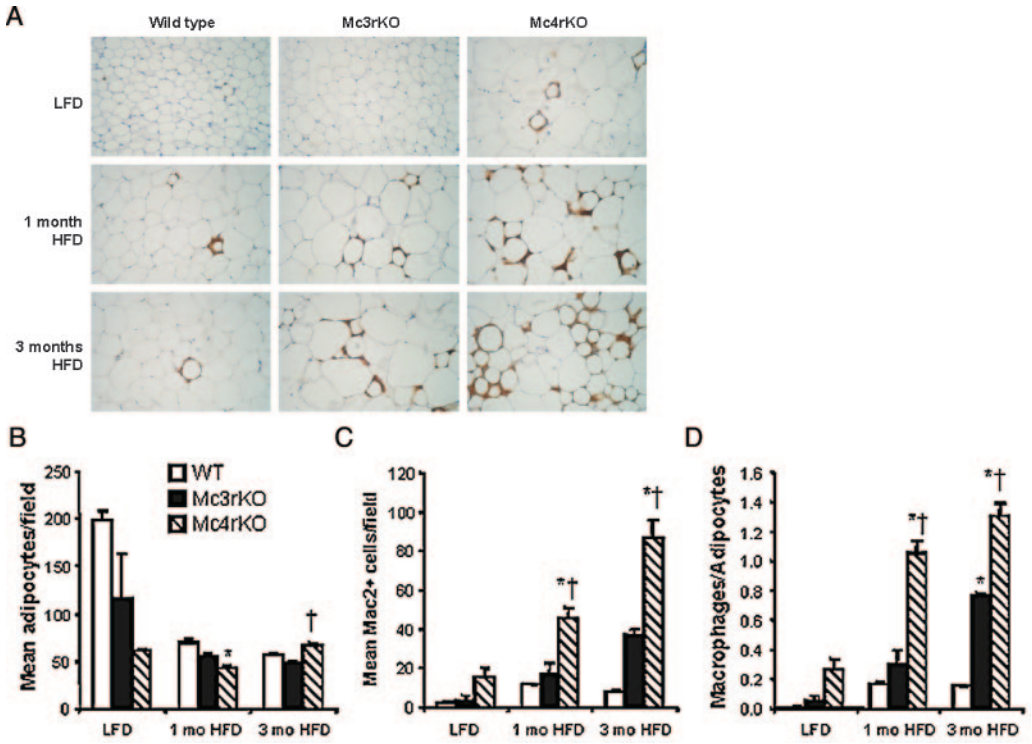

Figure 4: Macrophage infiltration and adipocyte hypertrophy are evident in Mc4rKO and are increased by HFD. (A) Representative images of retroperitoneal WAT sections stained for Mac-2, a macrophage-specific antigen, from WT, Mc3rKO, and Mc4rKO maintained on LFD or HFD for either 1 or 3 months. The total number of adipocytes (B) and Mac-2-positive cells (C) were counted from five random fields. The ratio of the number of macrophages relative to the total number of adipocytes (D) shows macrophage infiltration occurring in Mc3rKO and Mc4rKO after exposure to HFD, with a smaller increase in WT. * $p < 0.05$ compared with WT. † $p < 0.05$ compared with Mc3rKO.

study, macrophage infiltration in WAT tended to be higher in Mc4rKO compared with WT and Mc3rKO on LFD and was dramatically increased in Mc3rKO and Mc4rKO on exposure to HFD for 3 months. Mc3rKO, however, exhibited a delayed response to HFD such that, even after 3 months of maintenance on HFD, Mac-2-positive staining in WAT was not equivalent to that of Mc4rKO.

Obese leptin-deficient Lep^{ob}/Lep^{ob} mice exhibit high levels of *Cd68* gene expression and macrophage numbers in WAT (18), whereas Xu et al. (19) also reported an increased

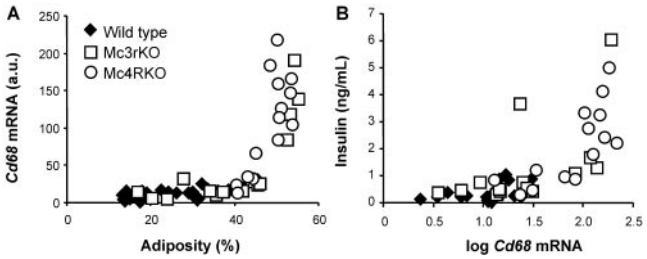

Figure 5: Relationship between the macrophage marker *Cd68* mRNA and adiposity (A) and *Cd68* mRNA and fasting insulin (B) in WT, Mc3rKO, and Mc4rKO.

expression of *Cd68*, *Emr1*, and *Mcp1* genes in adipose tissue of Lep^{db}/Lep^{db}. Adipose tissue *Cd68* gene expression is negatively associated with insulin sensitivity status in humans, and increasing insulin sensitivity by pioglitazone treatment is associated with a reduction in *Cd68* mRNA in adipose tissue (31). Reducing adipose tissue macrophage content through targeted deletion or inhibition of MCP1 or its receptor, C-C motif chemokine receptor-2, is associated with improved insulin sensitivity (20,32). Macrophage infiltration and inflammation are, therefore, factors associating obesity with insulin resistance. The attenuated inflammatory profile of Mc3rKO could be a factor contributing to mild insulin resistance observed in this model. Macrophages and a number of other cell types involved in the immune function and inflammation express Mc1r and Mc3r, with agonism of these receptors having an anti-inflammatory effect (33). However, although activation of Mc3r is anti-inflammatory, our data show inflammation associated with obesity is actually delayed in Mc3rKO. Whether Mc3r expressed on macrophages has a significant role in the inflammatory response to diet-induced obesity and whether loss of Mc3r protects against obesity-related inflammation was not determined in these studies. On the

Table 1. Summary of adipocyte cell number and size from retroperitoneal adipose tissue depot of WT, Mc3rKO, and Mc4rKO mice

Diet	Genotype	Adipocyte size (μL) (geometric mean)	Adipocyte number ($\times 10^3$)/mg tissue	Total number of adipocytes ($\times 10^6$)
LFD	WT	0.23 ± 0.05	8.8 ± 1.3	29.2 ± 5.3
	Mc3rKO	0.51 ± 0.05*	3.9 ± 1.4	22.1 ± 5.3
	Mc4rKO	0.78 ± 0.06*†	1.9 ± 1.6*	34.3 ± 5.8
HFD, 1 month	WT	0.71 ± 0.05	2.3 ± 0.1	20.5 ± 2.5
	Mc3rKO	0.93 ± 0.05*	1.6 ± 0.1	26.1 ± 2.9
	Mc4rKO	1.10 ± 0.06*	2.2 ± 0.2	64.1 ± 3.2*†
HFD, 3 months	WT	0.95 ± 0.04	1.8 ± 0.1	24.6 ± 2.7
	Mc3rKO	1.19 ± 0.06*	2.0 ± 0.1	52.3 ± 3.8*
	Mc4rKO	0.85 ± 0.06†	2.2 ± 0.1	64.3 ± 3.8*

WT, wild type mice; KO, knockout; Mc3rKo, Mc3r KO mice; Mc4rKo, Mc4r KO mice; LFD, low-fat diet; HFD, high-fat diet. Total adipocyte number was calculated by multiplying the number of adipocytes per milligram of tissue by the amount of triglyceride (fat mass in grams) per animal measured by nuclear magnetic resonance.
* $p < 0.05$ compared with WT mice.
† $p < 0.05$ compared with Mc3rKO mice.

other hand, Mc4rKO represents a new animal model of obesity where severe insulin resistance is coupled with inflammation.

The biology of adiponectin and its involvement in glucose homeostasis is complex and under active investigation. Adiponectin treatment attenuates alcohol- and obesity-related hepatomegaly and steatosis (34), whereas adiponectin KOs can exhibit insulin resistance that is exacerbated by diet (35). Serum adiponectin levels are regulated at the level of synthesis and secretion (36) and the rate of clearance from serum (25,37). The HMW isoforms of adiponectin are more relevant to insulin sensitivity status than total levels of adiponectin alone (38). We measured total serum adiponectin levels and, using Western blot, investigated the MMW and HMW isoforms. These results suggest differential effects of the obesity syndrome in Mc3rKO and Mc4rKO on serum adiponectin as factors contributing to differences in the insulin resistant phenotype. Mc3rKO and Mc4rKO on HFD exhibit weight gain and reduced WAT *Acrp30* mRNA expression; however, serum adiponectin levels in Mc3rKO remain comparable with WT. This may suggest differences in adiponectin clearance in obese Mc4rKO and Mc3rKO. Normal adiponectin levels could be a significant factor contributing to the mild insulin-resistant phenotype and mild hepatic steatosis observed in obese Mc3rKO fed HFD (14). Conversely, the reduction in adiponectin in Mc4rKO, observed for all MW forms, may contribute to the more severe insulin-resistant phenotype of Mc4rKO. However, insulin and glucose infusions have both been shown to reduce the HMW forms of adiponectin in mice (37). The more marked deterioration of glucose homeostasis associated with HFD may, therefore, also further aggravate hypoadiponectinemia in Mc4rKO.

Interventions that reduce reactive oxygen species reverse hypoadiponectinemia and improve diabetes, hyperlipidemia, and hepatic steatosis in obese mice (36). The *Acrp30* mRNA expression data, demonstrating a delayed decline in expression in Mc3rKO relative to Mc4rKO, may suggest differences in the effects of diet and genotype on oxidative stress in adipose tissue. A previous study reported an increase in adiponectin gene expression in mice after short-term exposure to HFD (39). However, in this study, a 1-month exposure to HFD did not result in significant changes in *Acrp30* mRNA in WT or Mc3rKO.

The relationship between the obesity phenotype of Mc4rKO and Mc3rKO and fat cell size is diet-dependent. On LFD, obesity in Mc4rKO and, to a lesser degree, Mc3rKO is distinguished by adipocyte hypertrophy, with no significant difference in the estimates of adipocyte number. Weight gain in Mc4rKO fed HFD diet is associated with adipocyte proliferation causing a rapid increase in the number of adipocytes, appearing to plateau within 1 month of exposure. In contrast, adipocyte hyperplasia in Mc3rKO is only observed with prolonged exposure to HFD. A capacity for triglyceride deposition into adipocytes and adipocyte proliferation have been suggested to protect against type 2 diabetes, allowing for partitioning of lipid to adipose and preventing lipotoxic effects in insulin target tissues and β-cells (40). Moreover, excess deposition of lipid metabolites in liver and muscle in situations of impaired adipocytes

proliferation is associated with insulin resistance (41). It seems reasonable, therefore, to speculate that a continued capacity for adipocyte proliferation and an expansion in adipocytes size may contribute to the mild insulin resistant phenotype of Mc3rKO.

For Mc4rKO, the effect of genotype and diet on insulin resistance is complex and tissue specific. Hepatic insulin resistance and hepatic steatosis develop in mildly obese Mc4rKO fed LFD, occurring independently of dietary fat content and presumably adipocyte hypertrophy (14,23). Mc4r, thus, appears to have a critical role in the normal regulation of liver metabolism and prevention of fatty liver disease in the mildly obese state. The relative contribution of hyperphagia and impaired autonomic or neuroendocrine inputs, such as leptin resistance and hypoadiponectinemia, to the fatty liver phenotype of Mc4rKO requires further investigation. Moreover, whether Mc4r signaling in Mc3rKO also functions to protect against severe fatty liver disease in the obese state is not clear.

In summary, we present data to show that in situations of adipocyte hypertrophy and hyperplasia associated with diet-induced obesity, Mc4rKO exhibit a severe inflammatory response in adipose tissue coupled with marked hyperinsulinemia and reduced serum adiponectin. The mild state of insulin resistance in severely obese Mc3rKO on HFD may be partially explained by the retention of relatively normal levels of adiponectin, combined with a delayed inflammatory response.

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